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# IDENTIFICATION OF THYROGLOBULIN mRNA SEQUENCES IN THE NUCLEUS AND THE CYTOPLASM OF CULTURED THYROID CELLS : A POST-TRANSCRIPTIONAL EFFECT OF THYROTROPIN

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SUMMARY. Cytoplasmic and nuclear RNA were isolated from porcine thyroid cells cultured with or without thyrotropin. After denaturation by glyoxal-DMSO treatment, the RNAs were analyzed on agarose gels, transferred onto nitrocellulose filters and hybridized with a [$^{32}$P]-labelled thyroglobulin cDNA clone. In the cytoplasmic RNA a single mature thyroglobulin mRNA molecule (9 Kb) was present whereas in the nuclear RNA several molecular species (24 to 3.3 Kb) were identified. In the nucleus only the 9 Kb mRNA was polyadenylated. In cytoplasmic preparations the level of mature thyroglobulin mRNA was two-fold higher when cells were cultured with thyrotropin. In nuclear preparations only the level of the mature transcript was affected by the presence of thyrotropin, increasing relative to the concentration of the higher molecular weight species. These results suggest a hormonal effect at the post-transcriptional level that may serve to stabilize the mature mRNA molecule.

Thyrotropin (TSH) has been shown to modulate thyroglobulin (Tgb) synthesis in cultured porcine thyroid cells (1) by regulating the Tgb mRNA level in the cytoplasm (2) and in the nucleus (3). In the cytoplasm, the relative Tgb mRNA content was kept steady by the presence of the hormone in the culture medium, as determined by Rot analysis using a single stranded cDNA probe obtained from purified Tgb mRNA (4). Absence of TSH during 4 days of culture caused a three fold decrease in the level of Tgb mRNA in the cytoplasm and a two-fold decrease in the nucleus (2,3). Subsequent addition of TSH resulted in an increase in the Tgb mRNA content suggesting hormonal action at the transcriptional or post-transcriptional levels (2). It is not known whether the hormone regulates Tgb mRNA transcription. However as recent studies have shown that the Tgb gene contains several introns (5,6), hormonal control may operate during the processing of its nuclear precursor transcripts. It has been propo-

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ABBREVIATIONS. Tgb : Thyroglobulin; TSH : Thyrotropin; Kb:Kilobase; bp : base pairs; DMSO : Dimethyl sulfoxide; EGTA : Ethylene glycol - bis - (β-amino-ethylether) N, N'-tetraacetic acid; SDS : Sodium dodecylsulfate.

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sed that hormonal regulation of specific genes may consist of a number of coordinated effects occuring at different steps of the expression of each gene (7). Other polypeptide hormones regulate gene expression by acting at transcriptional and post-transcriptional levels as was shown in the case of prolactin (8-11) which increased casein mRNA synthesis and stability. Thyrotropin releasing hormone (TRH) also induced the accumulation of prolactin mRNA in $GH_3$ cells (12) and promoted a rapid increase of the level of nuclear precursors of prolactin mRNA (13).

In the present study, a Tgb cDNA clone (14) was used to characterize Tgb sequences in preparations of RNA from nuclear and cytoplasmic fractions of TSH-treated or non-treated porcine thyroid cells in culture. In cytoplasmic RNA there is only one major detectable Tgb mRNA of 9 Kb. In nuclear RNA, larger Tgb transcripts are also present, but only the mature 9 Kb molecule is polyadenylated. In cells cultured in the absence of TSH, the quantity of mature Tgb transcript was lowered relative to the higher molecular weight species. If these larger transcripts are true precursors, these results suggest that TSH exerts a post-transcriptional control on Tgb gene expression possibly by allowing more efficient polyadenylation of the transcripts.

## MATERIAL AND METHODS

Cell culture. Porcine thyroid cells were isolated by a discontinuous trypsin-EGTA treatment (15) and cultured as previously described for 4 days as unstirred suspensions in the absence (control cells) or in presence of 1 mU/ml TSH (2).
Nuclei purification. Cells were centrifuged, washed first with a spinner salt solution and then with phosphate buffer salt solution. Cells were disrupted in 5% citric acid in a Dounce homogenizer with a tight-fitting pestle and nuclei were isolated according to Tsai et al (16).
Cytoplasmic and nuclear RNA extraction. Cytoplasmic RNA was purified from 27000 xg supernatant fractions by guanidinium chloride extraction (2). Nuclear RNA was obtained from purified nuclei homogenized in SDS-urea medium and extracted by phenol-chloroform-isoamylalcohol (16,17). Contaminant DNA was digested (16) by deoxyribonuclease I (Worthington) made ribonuclease-free by UMP-agarose and bentonite treatments (18). Residual proteins were eliminated by proteinase K digestion (16). Nuclear RNA containing polyadenylated sequences was isolated by oligo(dT) cellulose chromatography (19) with elution at room temperature, then at 60°C.
Preparation of cloned thyroglobulin [$^{32}$P]- cDNA probe. Ovine Tgb cDNA was cloned in PstI site of pBR 322 (14). Inserts from psTg 21A (1670 bp and containing one PstI site) and from psTg 11 (330 bp and containing poly (dA) tail) were obtained by digestion with PstI, separated by 4% polyacrylamide gel electrophoresis and eluted from the gel (20). These probes were labelled with [$^{32}$P]-dCTP (Amersham 400 or 800 Ci/mmole) by nick-translation and used for hybridization assays.
RNA agarose gel electrophoresis blotting and hybridization. Cytoplasmic and nuclear RNA were glyoxalated (deionized glyoxal, BDH) in a medium containing 50% DMSO (Sigma) according to Carmichael and Mc Master (21) then electrophoresed through agarose slab gels in a low ionic strength buffer (21). The size of nuclear Tgb mRNA species was evaluated in 0.5 % agarose gel. In 0.8% agarose the migration was not linearly related to the logarithm of the molecular weight in the high molecular weight regions. Ribosomal RNA from sheep thyroid glands used as markers were detected by acridine orange staining.

RNA were blotted onto nitrocellulose filter (Schleicher and Schüll BA 85) overnight (22). RNA fixed to the filter were hybridized with [$^{32}$P]-labelled probe as described (22). After extensive washing, the filter was exposed to film (Kodak X-O-mat AR5) with intensifying screen (Dupont de Nemours). Autoradiograms were recorded on densitometer (Gelman ACD 18).

## RESULTS

Tgb mRNA sequences were identified in cytoplasmic and nuclear RNA preparations from porcine thyroid cells cultured in presence or in absence of TSH. The RNAs were denatured by glyoxal and DMSO, analyzed by electrophoresis on agarose gels (see Methods) and transferred to nitrocellulose filters. To detect Tgb sequences, the RNA blots were hybridized with a mixture of 1.2 and 0.4 Kb PstI fragments of an ovine Tgb cDNA clone radiolabelled by nick-translation. This cloned cDNA represents the 3'end of Tgb mRNA (14). The cross-hybridization of ovine Tgb cDNA with porcine Tgb mRNA was previously demonstrated (2).

### Identification of Tgb mRNA sequences in the cytoplasmic fraction.

In both culture conditions, the probe gave a strong signal with an RNA species migrating at the position of polysomal Tgb 33 S mRNA obtained from sheep thyroid (Fig. 1A). Its size was evaluated as 9 Kb from a comparison of its electrophoretic migration with that of Hind III-cut λ phage DNA size markers (not shown). This size estimation is in good agreement with one made previously by analysis of purified Tgb mRNA on 99% formamide-acrylamide gels (19). Other Tgb mRNA sequences heterogeneous in size, gave a faint signal in the area of smaller molecular weight species likely corresponding to degradation products of 33 S mRNA. Densitometric analysis of autoradiograms (Fig. 1B) showed the concentration of Tgb mRNA in cytoplasmic RNA preparations to be at least two-fold higher in the cells treated with the hormone as compared to the cells cultured without it. The TSH/T ratio did not change with increasing RNA quantities. Cytoplasmic RNA from treated and untreated cell cultures did not contain Tgb sequences in molecules larger than the 9 Kb mature RNA.

### Identification of Tgb mRNA sequences in the nuclear fraction.

Nuclear RNA exhibited a minimum of 9 RNA species hybridizing to the Tgb cDNA probe. The size of each species reported in Table I is the average result of five different analyses on several nuclear RNA extracts. A 9 Kb transcript (Band E, Fig. 2A) migrated at the position of the mature cytoplasmic Tgb mRNA. Band A detectable in some experiments was not a well-defined band but a diffuse signal in the 24 Kb molecular weight region. The most abundant of the larger species, Band B (15 Kb), might correspond either to the accumulation of a transcript at a certain stage of processing or to several precursors with similar sizes. An increase in the concentration of the denaturing agent glyoxal (Fig. 2A) did not change the pattern of larger species indicating that they do not represent aggregates. Under these experimental conditions the nuclear

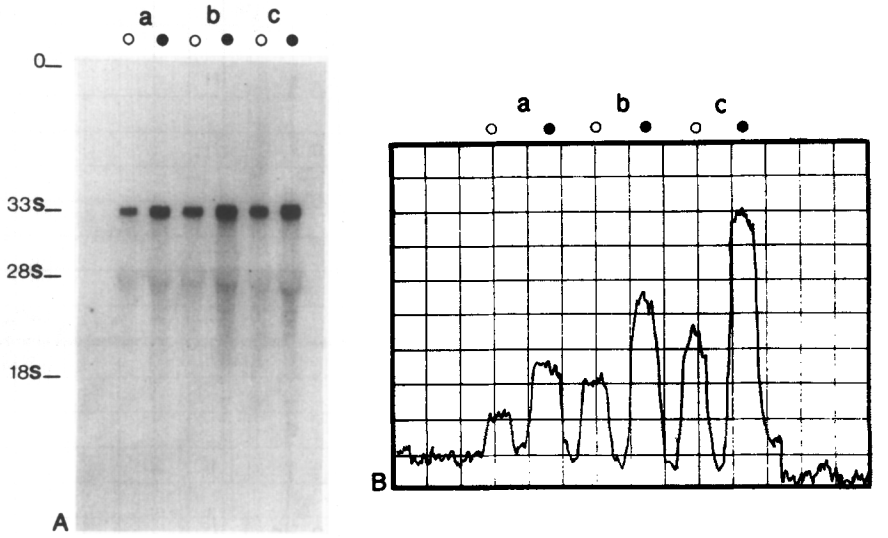


Figure 1. Identification of Tgb mRNA sequences from the cytoplasm of porcine thyroid cells.

A. Autoradiogram of cytoplasmic RNA analyzed on 1% agarose slab gel, transferred to nitrocellulose filter and hybridized with [$^{32}$P]-dCTP-labelled inserts from psTg 11 and psTg 21A (~5 x $10^6$ CPM/µg DNA). RNA from cells cultured without TSH (O) or with TSH (●). (a) 5 µg, (b) 10 µg, (c) 25 µg. Total sheep thyroid polysomal RNA containing 28 S and 18 S rRNA stained with acridine orange and Tgb 33 S mRNA were used as markers.

B. Densitometric profile of autoradiogram shown in Fig. 1 A.

RNA fraction contained significant amounts of RNA molecules whose sizes are smaller than the mature 9 Kb mRNA (G to I). These sequences could correspond to defined cleavage products of the precursor or mature molecules, possibly arising from defects in the splicing of certain introns or from nuclease digestion of highly susceptible regions within the transcripts. Band E, corresponding to the mature transcript, was more intense in the nuclear RNA from

Table I. Size evaluation of Tgb RNA sequences from the nuclear RNA of porcine thyroid cells.

| | SIZES OF NUCLEAR THYROGLOBULIN RNA MOLECULES (Kb) | |
|---|---|---|
| BANDS | EXTREME VALUES | AVERAGE VALUES |
| A | 24.3 - 25.7 | 25.0 |
| B | 13.6 - 16.6 | 15.1 |
| C | 13.0 - 13.6 | 13.3 |
| D | 10.9 - 11.7 | 11.3 |
| E (33 S) | 9.1 - 9.7 | 9.4 |
| F | 8.4 - 9.0 | 8.7 |
| G | 6.7 - 8.3 | 7.3 |
| H | 5.1 - 5.7 | 5.4 |
| I | 3.2 - 3.4 | 3.3 |

Nuclear RNAs were analyzed in 0.5% agarose slab gel (see Methods). Bands were identified as in Fig. 2A.

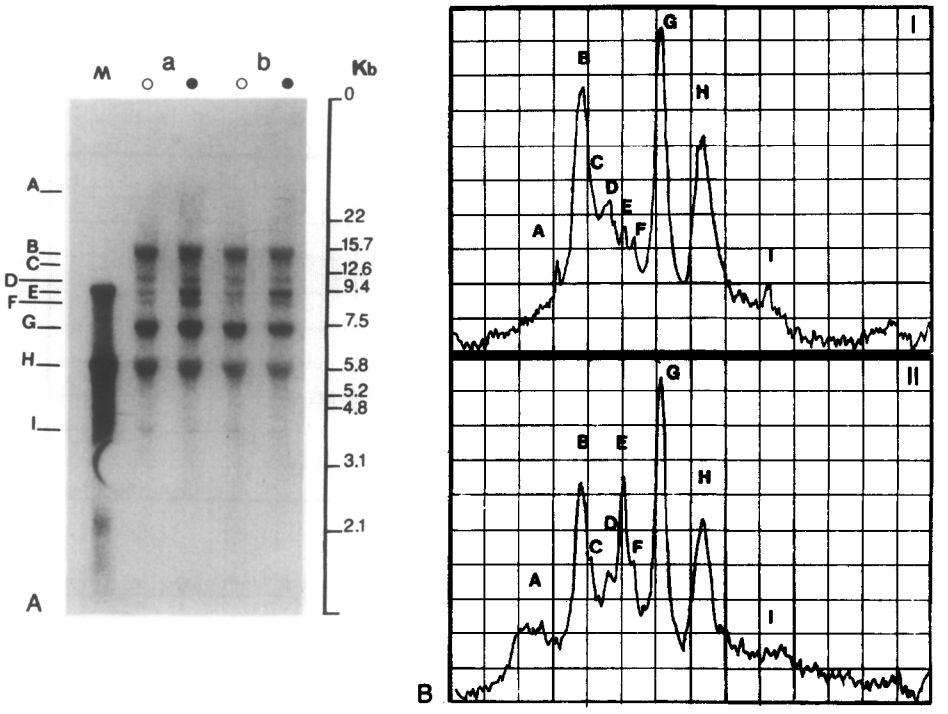


Figure 2. Identification of Tgb mRNA sequences from the nuclei of porcine thyroid cells.

A. Autoradiogram of nuclear RNA analyzed on 0.8% agarose slab gel, transferred to nitrocellulose and hybridized with [$^{32}$P]-dCTP-labelled insert from psTg 21A (~3 x $10^7$ CPM/µg DNA). RNA (50 µg) from cells cultured without TSH (○) or with TSH (●). (a) glyoxal 1M; (b) glyoxal 2.4M. 28 S and 18 S rRNA stained with acridine orange, Tgb 33 S mRNA (M), λDNA fragments digested by EcoRI or PvuI and DNA marker IV (Boehringer) hybridized with [$^{32}$P]-dCTP labelled fragments obtained by PvuI treatment, were used as markers.

B. Densitometric profiles of autoradiograms shown in Fig. 2A , I : from b (○) II : from b (●).

cells cultured with TSH (Fig. 2 A and B). No significant differences appeared for the higher molecular weight species.

Identification of polyadenylated transcripts in the nuclear RNA.

Total nuclear RNA from TSH-treated or control cells was fractionated by affinity chromatography on oligo (dT) cellulose and the eluted and flow-through fractions were analyzed in the same glyoxal gels. As shown in figure 3, the mature species E was found exclusively in the poly ($A^+$) fraction. The A, B, D, G and H species were mostly devoid of poly (A) sequences, although in some experiments they were present in low amounts in the poly ($A^+$) fraction suggesting that the Tgb mRNA precursors were polyadenylated at very low efficiency.

## DISCUSSION

Tgb mRNA species were identified and quantitatively estimated in the cytoplasmic and nuclear fractions isolated from porcine thyroid cells cultured

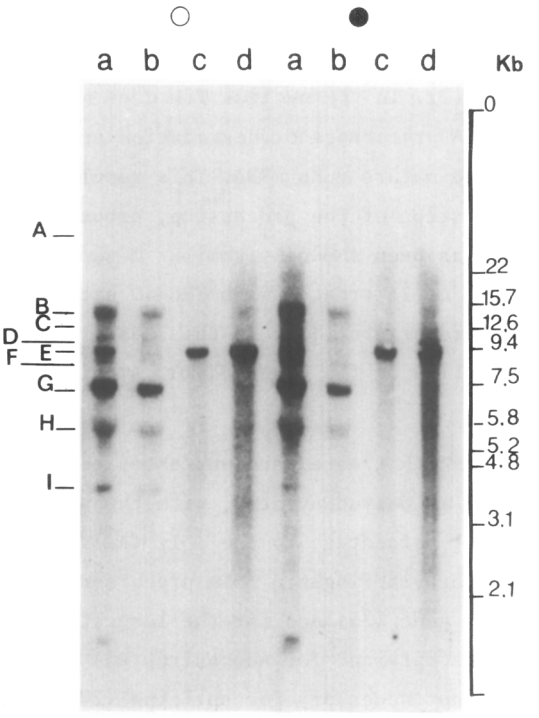


Figure 3. Identification of Tgb polyadenylated sequences from the nucleus of porcine thyroid cells.

Autoradiogram of nuclear RNA analyzed on 0,8 % agarose slab gel, transferred onto nitrocellulose and hybridized with [$^{32}$P]-dCTP-labelled insert from psTg 21A (~6 x $10^7$ CPM/µg DNA). RNA from cells cultured without TSH (○) or with TSH (●). (a) 50 µg total RNA; (b)~50 µg Poly ($A^-$) RNA; (c) 4 µg Poly ($A^+$) RNA; (d) 8 µg Poly ($A^+$) RNA.

with or without TSH. In the cytoplasm there was only one major molecular weight species, the mature Tgb mRNA (9 Kb). Higher molecular weight species present in the nucleus were not observed in the cytoplasm suggesting that processing of these molecules must be completed before their transport to the cytoplasm as it has been generally observed for mRNA precursor molecules, e.g. for ovalbumin mRNA in the chicken oviduct (23). Among the nuclear mRNA sequences we detected transcripts ranging from 24 to 10 Kb which may be precursors of the mature Tgb mRNA. Recently a genomic DNA fragment coding for the 5'end of rat Tgb mRNA has been shown to contain very large introns representing 93% of the fragment (5). Studies of the human Tgb gene have shown that it may span more than 100 Kb (6). We have not observed such high molecular weight transcripts. They may be present but in undetectable amounts as described for the globin precursors (24) or the exon sequences may be so short as to be insufficient for stable hybridization with the probe. As a matter of fact, it has been observed that the 3'end of human Tgb gene (6) contains short exons (130-200 bp) embedded in very large introns (15000 bp and more).

In the cytoplasm, the relative content of the mature Tgb mRNA (9 Kb) was at least two-fold higher in TSH-treated cells. This result is in good agreement with previous data (2). In the nucleus TSH does not influence the content of putative Tgb mRNA precursors or degradation products but clearly promotes accumulation of the mature 9 Kb mRNA. This result suggests that the hormone regulates the last steps of the processing, probably by stabilization of the mature mRNA. It has been shown by similar methods that the induction of prolactin mRNA in $GH_3$ cells treated with TRH is accompanied by accumulation of prolactin mRNA precursors (13). In this case, the hormone may act by stimulating transcription of the gene and/or stabilizing the precursor molecules.

In nuclear RNA from both treated and untreated cells, it is mainly the mature Tgb mRNA that is polyadenylated, with the putative precursors contributing only a small percentage to the poly ($A^+$) fraction. Similar behaviour was observed for vitellogenin mRNA precursors which are also transcribed from a large gene (25) and for the largest globin mRNA precursor (26). However results obtained for Adenovirus RNA processing show that polyadenylation, while not necessary for splicing (27), usually precedes the removal of the last introns (28). However, it was observed that all the precursors of ovalbumin mRNA, ovomucoid mRNA (29) and prolactin mRNA (13) are polyadenylated.

The Tgb gene is known to be very large (6) and we observed the absence of high molecular weight precursors and the accumulation of non polyadenylated incompletely processed molecules. So we can hypothesize that the primary transcripts of the Tgb gene are probably processed while their transcription is still proceeding. Such a mechanism has been observed for Adenovirus late transcripts (30). Bands A, C, D and mainly B could be accumulated molecules after termination of transcription when processing was not complete. Polyadenylation occuring at a low percentage for these molecules could be the signal of their final splicing resulting in the species E and stabilizing this mature mRNA (see 31). One step of TSH action might be the stimulation of polyadenylation of precursors thereby activating the last steps of the messenger molecule maturation.

In conclusion, we have shown that in cultured thyroid cells, TSH does not alter the molecular weight pattern or composition of nuclear Tgb transcripts but promotes preferential accumulation of the mature polyadenylated molecules. TSH seems to act mainly at the post-transcriptional level, likely by stabilizing the mature mRNA in the nucleus and the cytoplasm.

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REFERENCES

1. Chabaud,O., Chebath,J., Giraud,A. and Mauchamp,J. (1980) Biochem.Biophys. Res.Commun., 93, 118-126.
2. Chebath,J., Chabaud,O. and Mauchamp,J. (1979) Nucl.Acids Res., 6, 3353-3368.
3. Chabaud,O., Chebath,J. and Mauchamp,J. (1981) Reprod.Nutri.Develop., 21, 237-245
4. Chabaud,O., Chebath,J. and Jacquet,M. (1980) Eur.J.Biochem. 104, 125-136.
5. Christophe,D., Pohl,V., Van Heuverswijn,B., De Martynoff,G., Brocas,H. Dumont,J.E., Pastels,J.L. and Vassart,G. (1982) Biochem.Biophys.Res.Commun., 105, 1166-1175.
6. Van Omnem,G.J.B., Arnberg,A.C., Baas, F., Brocas,H., Sterk,A., Tegelaers, W.H.H., Vassart,G. and de Vijlder, J.J.M. (1983) Nucl.Acids Res.,11, 2273-2285.
7. Rosen,J.M., Matusik,R.J., Richards D.A., Gupta,P. and Rodgers,J.R. (1980) Recent Progress in Hormone Research, R.O.Greep Ed., Vol.36 p.157, Academic Press, New York.
8. Houdebine, L.M. (1976) Eur.J.Biochem., 68, 219-225.
9. Houdebine,L.M., Devinoy,E. and Delouis,C. (1978) Biochimie, 60, 57-63.
10. Teyssot,B. and Houdebine, L.M. (1980) Eur.J.Biochem., 110, 263-272.
11. Guyette,W.A., Matusik,R.J. and Rosen J.M. (1979) Cell, 17, 1013-1023.
12. Evans,G.A., David,D.N. and Rosenfeld M.G. (1978) Proc.Natl.Acad.Sci. U.S.A. 75, 1294-1298.
13. Potter,E., Nicolaisen, A.K., Ong,E.S., Evans, R.M. and Rosenfeld, M.G. (1981) Proc.Natl.Acad.Sci. USA, 78, 6662-6666.
14. Chebath,J., Tosta,Z.T., Chabaud,O. and Perricaudet,M. (1982) Molec.Biol. Rep., 8, 149-156.
15. Mauchamp,J., Margotat,A., Chambard,M., Charrier,B., Rémy,L. and Michel-Béchet,M. (1979) Cell and Tissue Res., 204, 417-430.
16. Tsai,S.Y., Roop,D.R., Tsai,M.J., Stein,J.P., Means,A.R. and O'Malley, B.W. (1978) Biochemistry, 17, 5773-5780.
17. Roop,D.R., Nordström,J.L., Tsai,S.Y., Tsai,M.I. and O'Malley, B.W. (1978) Cell, 15, 671-685.
18. Grady,L.J., Campbell, W.P. and North,A.N. (1980) Anal.Biochem., 101, 118-122.
19. Chebath,J., Chabaud,O., Bergé-Lefranc,J.L., Cartouzou,G. and Lissitzky,S. (1977) Biochem.Biophys.Res.Commun., 79, 267-273.
20. Maniatis,T. and Efstratiadis,A. (1980) Methods in Enzymology, Grossman,L. and Moldave,K. Eds. Vol.65 part I, p.299-304, Academic Press, New York.
21. Carmichael,G.G. and Mc Master, G.K. (1980) Methods in Enzymology, Grossman,L. and Moldave,K. Eds, Vol.65, part I, p.380-391, Academic Press, New York.
22. Thomas,P. (1980) Proc.Natl.Acad.Sci.U.S.A., 77, 5201-5205.
23. Tsai,M.J., Tsai,S.Y. and O'Malley,B.W. (1979) Science, 204, 314-316.
24. Reynaud,C.A., Imaizumi-Scherrer, M.T. and Scherrer,K. (1980) J.Mol.Biol. 140, 481-504.
25. Jost,J.P., Pehling,G., Ohno,T. and Cozens,P. (1978) Nucl.Acids Res., 5, 4781-4793.
26. Bastos,R.N. and Aviv,H. (1977) Cell, 11, 641-650.
27. Zeevi,M., Nevins,J.R. and Darnell,Jr., J.E. (1981) Cell, 26, 39-46.
28. Nevins,J.R. and Darnell Jr., J.E. (1978) Cell, 15, 1477-1493.
29. Tsai,M.J., Ting,A.C., Nordström,J.L., Zimmer,W. and O'Malley, B.W. (1980) Cell, 22, 219-230.
30. Mariman,E.C.M., Van Beek-Reinders,R.J. and Van Venrooij,W.J. (1983) J.Mol.Biol., 163, 239-256.
31. Littauer,U.Z. and Soreq.H. (1982) Progress in Nuc.Acid Res. and Mol. Biol., Vol.27, pp 53-83, Academic Press, New York.